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Simvastatin inhibits PDGF-induced DNA synthesis in human glomerular mesangial cells

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Simvastatin inhibits PDGF-induced DNA synthesis in human glomerular mesangial cells. Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase ameliorate glomerular pathology and renal dysfunction in different models of glomerular disease. This effect has generally been attributed to a decrease in the circulating levels of cholesterol. Focal or diffuse mesangial cell proliferation is a common feature of glomerular pathology. There is now evidence from studies *in vitro* and *in vivo* that platelet-derived growth factor (PDGF) is an important mediator of glomerular hypercellularity. The activity of HMGCoA reductase has previously been shown to be a requirement for cell growth. In the present study, we examined the effect of simvastatin, an HMGCoA reductase inhibitor, on PDGF-induced DNA synthesis and PDGF B chain gene expression in human glomerular mesangial cells. In addition, we investigated the effect of simvastatin on phospholipase C (PLC) and protein kinase C (PKC) activation stimulated by PDGF. We demonstrate that treatment of the cells with simvastatin completely inhibits PDGF-induced DNA synthesis. This inhibition is reversed by mevalonate but not by cholesterol or farnesol, two major metabolites of the mevalonate pathway. On the other hand inhibition of HMGCoA reductase does not influence PDGF-induced activation of PLC and PKC, or PDGF B chain gene expression. These data suggest that simvastatin acts at a late step in the PDGF mitogenic pathway without interfering with other early cellular responses elicited by this growth factor. These studies also raise the possibility that the ameliorative effect of HMGCoA reductase inhibitors on glomerular pathology may be mediated, at least in part, by a direct cellular effect.

Progressive renal diseases, particularly those characterized by severe proteinuria, are almost invariably associated with elevated plasma levels of lipids [1, 2]. The associated hypercholesterolemia has been incriminated as an aggravating factor that accelerates the development of glomerulosclerosis [3]. Several studies in experimental animals with nephrotic syndrome or with various degrees of renal ablation have documented the deleterious effect of cholesterol feeding on renal and specifically glomerular pathology [3]. Moreover, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, the rate limiting enzyme in the cholesterol biosynthetic pathway, ameliorate the structural and functional changes seen in these models [4–6]. The beneficial effects of these cholesterol lowering drugs have generally been attributed to the decrease in circulating levels of cholesterol. Certain forms of human as well as experimental models of glomerular injury are characterized by focal or diffuse mesangial cell proliferation [7]. Presently, there is evidence that activation and proliferation of this population of glomerular cells may represent an important step in the development of diffuse glomerulosclerosis [8]. The precise mechanisms that lead to mesangial cell proliferation are incompletely understood. Platelet-derived growth factor (PDGF) could play a key role in this context. PDGF is a potent mitogen for mesangial cells *in vitro* [9] and there is evidence that this growth factor and its receptor are upregulated in experimental and human glomerular diseases [10–12]. Several recent observations suggest an important role for cholesterol or other metabolites of the mevalonate pathway in the signal transduction mechanisms involved in cell transformation and growth [13–15]. Inhibition of mevalonate production in cells treated with HMGCoA reductase inhibitors [13, 15] or in mutant cells lacking HMGCoA synthase [16] causes an arrest of the cell cycle in G1 phase that is not overcome by cholesterol, suggesting that intermediate(s) in this metabolic pathway may have a central role in the regulation of the cell cycle. These observations raise the possibility that the effect on glomerular pathology observed in response to these drugs may result, at least in part, from a direct action at the cellular level. In our study, we investigated the effect of an HMGCoA reductase inhibitor, simvastatin, on DNA synthesis and autoinduction of PDGF B chain gene expression in human glomerular mesangial cells stimulated by PDGF. In addition we investigated the effect of simvastatin on two early events in the PDGF signal transduction pathway, phospholipase C (PLC) and protein kinase C (PKC) activation.

Methods

Cell culture and characterization

Normal human kidney tissue unsuitable for transplantation was used to culture mesangial cells from outgrowths of collagenase-treated glomeruli. Mesangial cells were extensively characterized by electron microscopy and immunohistochemical staining as previously described [9]. The cells were grown in Waymouth's medium containing 17% fetal calf serum. In all the experiments the cells were used between passages six and ten.

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Measurement of DNA synthesis

DNA synthesis was measured as the incorporation of [^{3}H]-thymidine into trichloroacetic acid (TCA) insoluble material. Confluent mesangial cells in 24 well dishes were made quiescent by placing them in serum free Waymouth's medium for 72 hours. For studying the effect of HMGCoA reductase inhibitor simvastatin, the cells were preincubated with simvastatin or vehicle dimethylsulfoxide at the indicated concentrations for 18 hours before the experiment. Human recombinant PDGF BB (12 ng/ml) was then added to quadruplicate wells for 28 hours. During the last four hours of the incubation period the cells were pulsed with [^{3}H]-thymidine (1 μCi/ml, New England Nuclear, Boston, Massachusetts, USA). The medium was then removed, the cells were washed twice in ice cold 5% TCA and then incubated in 5% TCA on ice for five minutes. The cells were solubilized by adding 0.7 ml of 0.25 N NaOH in 0.1% sodium dodecyl sulfate (SDS). Half milliliter aliquots were then neutralized and counted in scintillation fluid.

RNA isolation and solution hybridization assay

Human glomerular mesangial cells grown to confluence in 100 mm petri dishes were made quiescent by placing them in serum-free Waymouth's medium for three days. The cells were treated for the last 18 hours with simvastatin, then PDGF BB (12 ng/ml) was added. At the end of the incubation the cells were lysed in guanidine isothiocyanate buffer at the indicated time points. Sodium-N-laurylsarcosine to a final concentration of 0.5% and 0.35 g/ml of cesium chloride (CsCl) were added and the cell homogenates were layered on a 1.2 ml cushion of 5.7 M CsCl, 0.1% EDTA and centrifuged for 18 hours at 100,000 × g at 20°C. The supernatant was carefully discarded and the pellet containing the RNA was resuspended in 5 mM sodium citrate buffer and extracted with chloroform/butanol. 0.3 M sodium acetate followed by 2.5 volumes of absolute ethanol were added and the RNA precipitated from the aqueous phase after centrifugation was resuspended in sterile water, quantified by spectrophotometry and stored at −80°C. A ribonuclease protection assay was used to determine PDGF B chain mRNA expression. The cDNA template was constructed by cloning a 960 bp BanII-BanII restriction fragment encompassing portion of exons 6 and 7 of the c-*sis* gene into the SmaI site of plasmid pT7-2. To generate the single stranded ^{32}P labeled RNA probe, the plasmid was linearized and transcription was carried out using T7 RNA polymerase (Promega) in the presence of 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 40 mM dithiothreitol, 40 U/ml RNasin, 0.5 mM each of ATP, GTP and CTP, 12 μM UTP and 50 μCi of ^{32}P-UTP (800 Ci/mmol, Amersham) at 37°C for one hour. This was followed by the addition of RNase free DNase (Promega) and another 15 minutes incubation to digest the DNA template. After repeated extraction with phenol chloroform and precipitation in ethanol, the RNA pellet was resuspended in hybridization buffer consisting of 80% formamide. The labeled RNA probe (1×10^6 cpm) was hybridized at 50° to 100 μg of total RNA prepared from mesangial cells. After hybridization the samples were digested with RNase A (50 μg/ml) and RNase T_1 (2 μg/ml) followed by the addition of proteinase K to inactivate the remaining RNase. After extraction in phenol/chlorophorm the samples were precipitated in ethanol and redissolved in loading buffer containing 90% formamide and separated on 6% polyacrylamide/urea gel. Gels were exposed to Kodak Xomat films with intensifying screens at −70°C.

Phosphatidylinositol hydrolysis

Confluent mesangial cells in 12 well dishes were placed in inositol free serum free RPMI 1640 containing 3 μCi/ml of 2[^{3}H]-myoinositol for 48 hours. In the last 18 hours, the cells were treated with simvastatin at the indicated concentrations. After a 15 minute preincubation with 15 mM LiCl at 37°C, PDGF BB (12 ng/ml) was added to triplicate wells, with or without the HMGCoA reductase inhibitor, and incubated for five minutes. The cells were harvested in 5% trichloroacetic acid (TCA) and ether extracted to remove the TCA. The samples were then loaded onto 2 ml AG1X8 anion exchange columns (Biorad). The columns were washed with 10 ml of water and 10 ml of 5 mM sodium tetraborate. Inositol phosphates were eluted with 10 ml of 0.8 M ammonium formate in 0.1 M formic acid. Five milliliters of this fraction were mixed with scintillation fluid and counted in a beta counter.

Protein kinase C assay

Confluent mesangial cells were made quiescent by placing them in serum free medium for 48 hours. In the last 18 hours, the cells were preincubated with simvastatin (10 μM) and then stimulated with PDGF BB (12 ng/ml) for 15 minutes. The cells were washed twice with phosphate buffered saline and lysed in homogenization buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X100, 25 μg/ml aprotinin, 25 μg/ml leupeptin) at 4°C. The cell lysate was centrifuged at 10,000 × g for 30 minutes at 4°C. Protein kinase C was partially purified using the DE-52 (DEAE) column. The protein concentration in the eluate was measured by the Bio-Rad method. Protein kinase C activity was determined by measuring phosphotransferase activity to a synthetic peptide from myelin basic protein in the presence of phorbol ester, phosphatidylserine and ^{32}P labeled ATP. A pseudosubstrate inhibitor was used to determine the nonspecific activity. Phosphotransferase activity was expressed as pmoles of ^{32}P incorporated into the synthetic peptide/5 min. Reagents used were from a commercial kit (BRL).

Results

Effect of simvastatin on PDGF-induced DNA synthesis

First, we examined the effect of HMGCoA reductase inhibition on PDGF-induced DNA synthesis in human glomerular mesangial cells. When the cells were pretreated with simvastatin, PDGF-induced ^{3}H-thymidine incorporation into DNA was inhibited in a dose dependent manner (Fig. 1). The inhibitory effect was already detectable at 1 μM and was maximal at 10 μM. To exclude a toxic effect of simvastatin, cell viability was checked by trypan blue exclusion. Under the same conditions in which simvastatin completely inhibited DNA synthesis cell viability was 94.9% + 3.3%. To demonstrate that the inhibition of DNA synthesis was due to inhibition of the HMGCoA reductase, we investigated the effect of simvastatin on DNA synthesis in the presence of mevalonate. The ability of mevalonate to completely restore PDGF-induced DNA synthesis (Fig. 2) indicates that the inhibitory effect is, indeed, related to

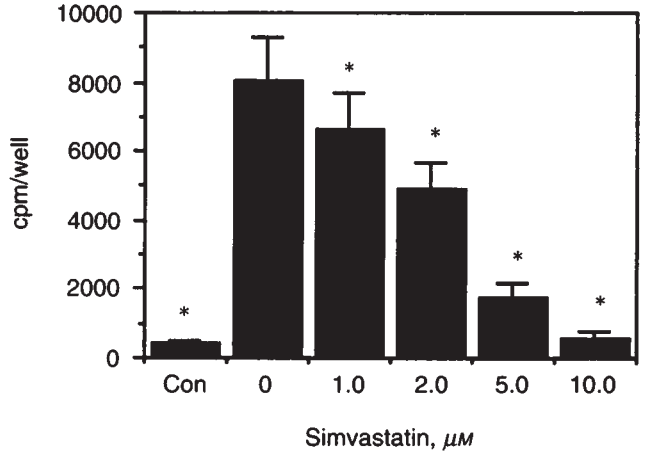

Fig. 1. *Effect of simvastatin on PDGF-induced DNA synthesis.* Confluent quiescent mesangial cells were pretreated with the indicated concentrations of simvastatin for 18 hours before the addition of PDGF (12 ng/ml). DNA synthesis was measured as the amount of [^{3}H]-thymidine incorporated into trichloroacetic acid insoluble material as described in **Methods**. Data represent mean ± SEM of 5 separate experiments, each done in quadruplicate wells. *$P < 0.05$ compared with PDGF.

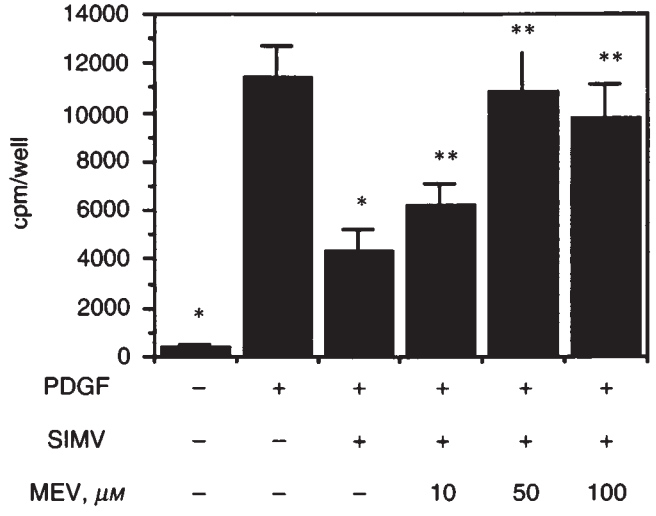

Fig. 2. *Effect of simvastatin and mevalonate on PDGF-induced DNA synthesis.* Confluent quiescent mesangial cells were preincubated with simvastatin (5 μM) and the indicated concentration of mevalonate for 18 hours before the addition of PDGF (12 ng/ml). DNA synthesis was measured as the amount of [^{3}H]-thymidine incorporation into trichloroacetic acid insoluble material as described in **Methods**. Data represent mean ± SEM of three separate experiments each done in triplicate. *$P <$ 0.01 compared with PDGF, **$P < 0.01$ compared with PDGF + simvastatin.

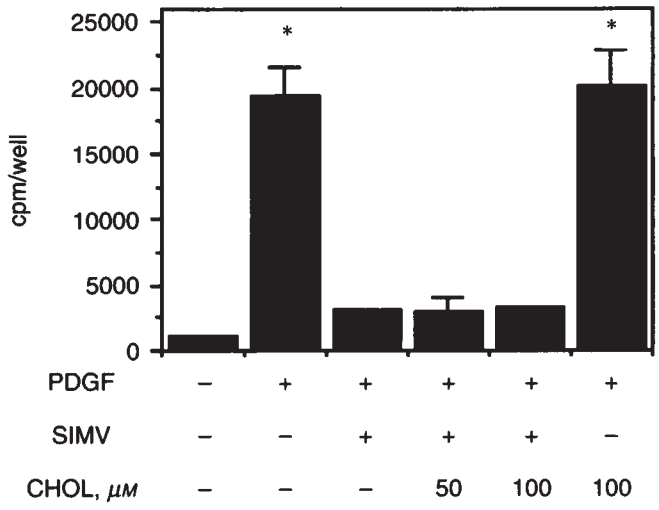

Fig. 3. *Effect of simvastatin and cholesterol on PDGF-induced DNA synthesis.* Confluent quiescent mesangial cells were preincubated with simvastatin (5 μM) and cholesterol at the indicated concentration for 18 hours. The cells were then stimulated with PDGF (12 ng/ml) and DNA synthesis was measured as incorporation of [^{3}H]-thymidine into trichloroacetic acid precipitable material as described in **Methods**. Data represent mean ± SEM of four separate experiments each done in quadruplicate. *$P < 0.01$ compared with PDGF.

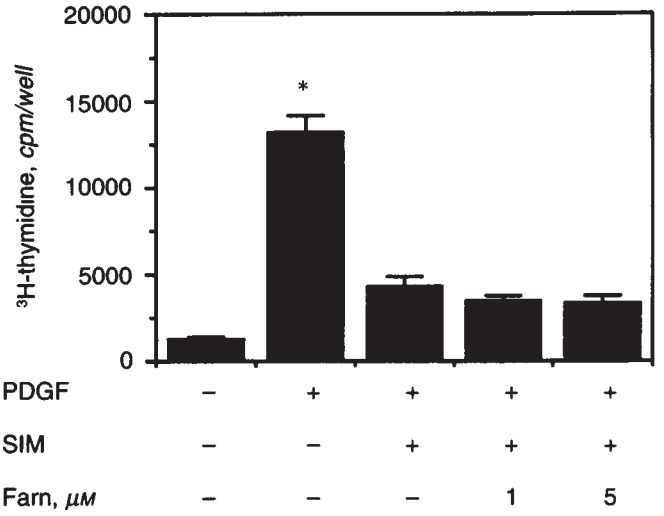

Fig. 4. *Effect of simvastatin and farnesol on PDGF-induced DNA synthesis.* Confluent quiescent mesangial cells were pretreated with simvastatin (5 μM) and farnesol at the indicated concentration for 18 hours. The cells were then stimulated with PDGF (12 ng/ml) and DNA synthesis was measured as the amount of [^{3}H]-thymidine incorporated into trichloroacetic acid precipitable material as described in **Methods**. Data represent mean ± SEM of three separate experiments each done in quadruplicate. *$P < 0.01$ compared with PDGF.

the inhibition of the mevalonic acid pathway. On the other hand addition of cholesterol did not overcome the inhibition caused by simvastatin as shown in Figure 3. We next examined if farnesol could reverse the inhibitory effect of simvastatin. Farnesol is directly involved in protein isoprenylation and is the precursor of all other nonsterol isoprenes including dolichol and ubiquinone. The addition of 5 μM Farnesol did not reverse the effect of simvastatin (Fig. 4). Higher concentrations of farnesol actually resulted in a reduction of ^{3}H-thymidine uptake by the cells (data not shown).

Effect of simvastatin on PDGF-induced PDGF B chain gene expression

We have previously demonstrated that PDGF is a potent inducer of PDGF B chain gene expression in mesangial cells [9].

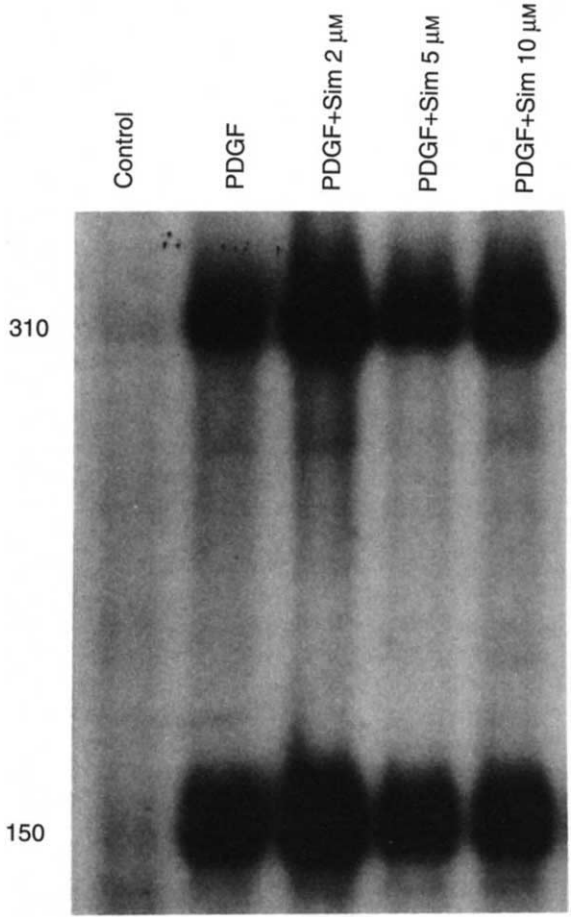

Fig. 5. *Effect of simvastatin on PDGF-induced PDGF B chain mRNA.* Quiescent mesangial cells were preincubated for 18 hours with simvastatin at the indicated concentrations. PDGF (12 ng/ml) was then added, the cells were harvested after 3 hours and RNA was isolated as described in **Methods**. One hundred micrograms of total RNA were hybridized with a uniformly labeled c-*sis* RNA probe. Following digestion with RNase A and T1, the two protected fragments of 150 and 310 bp were electrophoresed on a denaturing polyacrylamide gel and detected by autoradiography.

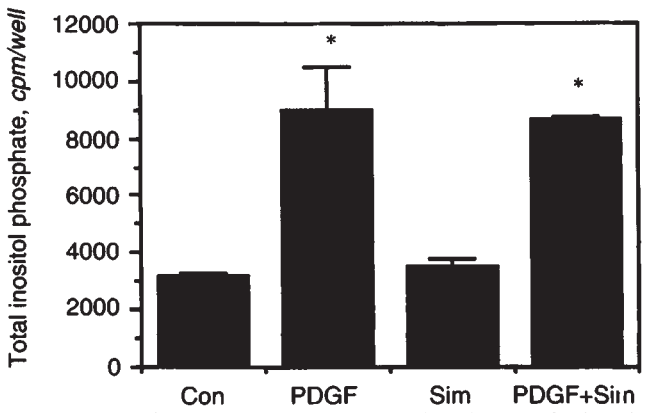

Fig. 6. *Effect of simvastatin on PDGF-induced inositol phosphate production.* Confluent mesangial cells were incubated for 48 hours in serum-free inositol-free RPMI 1640 and labeled with myo-2^{3}H-inositol for the same period. The cells were pretreated with simvastatin (10 μM) for 18 hours. PDGF was then added to the cells for 10 minutes. Inositol phosphates were extracted and analyzed as described in **Methods**. Data represent mean ± SEM of six observations from two separate experiments. *$P < 0.01$ compared with control.

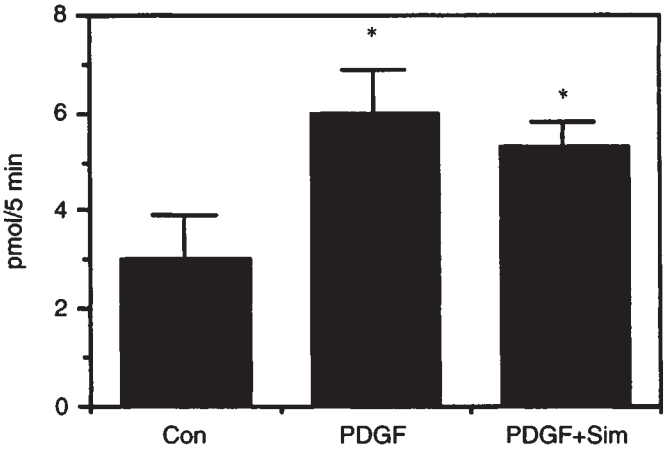

Fig. 7. *Effect of simvastatin on PDGF-induced PKC activation.* Quiescent confluent mesangial cells were pretreated with 10 μM simvastatin for 18 hours and then stimulated with 12 ng/ml of PDGF BB for 15 minutes. The cells were then lysed and the PKC activity was measured as described in **Methods**. Data represent mean ± SEM of six observations from two separate experiments. *$P < 0.01$ compared with control.

The induction of this gene is closely linked to mitogenesis since growth factors that stimulate DNA synthesis in mesangial cells also increase PDGF B chain mRNA levels [17]. We investigated the effect of simvastatin on PDGF-induced PDGF B chain gene expression. At concentrations that inhibited DNA synthesis, simvastatin had no effect on the induction of PDGF B chain mRNA (Fig. 5).

Effect of simvastatin on PLC and PKC activity

To determine if simvastatin interferes with early transmembrane signaling events in the PDGF mitogenic pathway, we examined its effect on the activation of PLC and PKC induced by PDGF. As shown in Figure 6, PLC activity measured as the amount of intracellular inositol phosphates, is unaffected by simvastatin. PKC activation in response to PDGF was also not inhibited by simvastatin at concentrations that completely inhibit DNA synthesis (Fig. 7). Therefore, inhibition of mevalonate synthesis does not influence the early steps in PDGF signalling pathways. The magnitude of PLC and PKC activation are similar in control cells and in cells treated with simvastatin suggesting that this agent does not interfere with PDGF binding to its receptor. Moreover, this observation excludes a toxic effect of simvastatin on mesangial cells.

Discussion

In the present study, we demonstrate that treatment of glomerular mesangial cells with simvastatin inhibits PDGF-induced DNA synthesis in a dose dependent manner. This inhibition can be reversed by the addition of mevalonate but not cholesterol or farnesol suggesting the importance of other product(s) of the mevalonate pathway in PDGF-induced DNA synthesis. HMGCoA reductase inhibition had no effect on three other events induced by PDGF: activation of PLC and PKC and autoinduction of PDGF B chain mRNA. There have been

conflicting reports regarding the necessity of HMGCoA reductase activity for PDGF-induced mitogenic response. While Fairbanks, Witte and Goodman reported a close relationship between the mevalonate pathway and mitogenesis in human fibroblasts [13], Vincent, Walfert and Merler showed that the mitogenic signalling pathway is unaffected by HMGCoA reductase inhibition [18]. The precise mechanisms by which the mevalonate pathway influences cell growth are not yet completely clear and the eventual mediator has not been identified. As we have demonstrated, cholesterol is not an absolute requirement. Besides cholesterol there are other products of the mevalonate pathway that are potential candidates for this role. Ubiquinone and heme are terminal products of the mevalonate pathway [19]. They are essential for mitochondrial respiratory function, therefore the reduction of their intracellular concentration may interfere with vital cell functions [19]. Moreover, it has been demonstrated that the concentration of HMGCoA reductase inhibitor that completely blocks cholesterol production only slightly inhibits ubiquinone synthesis [19]. Dolichol, another terminal product of the mevalonate pathway, plays a key role in protein glycosylation [20]. Although exogenous dolichol is ineffective in restoring growth of cells in which mevalonate synthesis was blocked [15], biologically active dolichol must be phosphorylated and this does not readily occur when exogenous dolichol is added to cells in culture [20]. Besides dolichol, farnesyl and geranyl isoprenylated proteins are other important candidates. One of the isoprenylated proteins is the $p21^{ras}$, the product of the ras oncogene [21]. Ras can induce DNA synthesis as well as transient morphological transformation when microinjected into quiescent cells [22, 23]. Farnesilation of the $p21^{ras}$ is an absolute requirement for its transforming activity [14, 24]. In NIH 3T3 cells, microinjection of anti-ras antibodies inhibits DNA synthesis induced by PDGF [25]. Ras has been shown in different systems to induce phosphatidyl inositol hydrolysis and PKC activation [26, 27]. Moreover, PKC activity plays an important role in mediating the mitogenic effect of ras proteins [26, 28]. Our finding that simvastatin did not interfere with PLC or PKC activation suggests, albeit indirectly, that the effect of simvastatin is not due to prevention of ras protein activation. The addition of farnesol, the precursor of all nonsterol isoprenes, did not reverse the effect of simvastatin on PDGF-induced DNA synthesis. This observation contrasts with recent studies where inhibition of serum-stimulated DNA synthesis by lovastatin in mesangial cells isolated from Zucker rats was dependent on farnesol [29]. This difference may be species specific or may be due to the different agonists used bovine serum versus PDGF since the small amount of PDGF present in bovine serum cannot account for its mitogenic effect [30]. Since only phosphorylated farnesol can enter the pathway, it is conceivable that exogenous farnesol can be phosphorylated by rat but not human mesangial cells under the incubation conditions utilized in our study. Other molecules that precede farnesol in the mevalonate pathway may also be essential for PDGF-induced DNA synthesis. One likely candidate is isopentenyl pyrophosphate. This intermediate of the mevalonate pathway is the precursor for isopentenyladenine. Previous studies have shown that isopentenyladenine can effectively reverse DNA synthesis after HMGCoA reductase inhibition [31, 32]. This compound is essential for cells in late G1 to carry out S phase DNA replication [32]. However, the addition of isopentenyladenenine did not restore DNA synthesis inhibited by simvastatin (data not shown). In rat mesangial cells, isopentenyladenine also did not reverse the inhibitory effect of lovastatin on DNA synthesis [30]. These findings do not exclude a role for other derivative(s) or intermediates of the mevalonate pathway in mediating PDGF-induced DNA synthesis. Regardless of the mechanism by which the mevalonate pathway is involved in the regulation of DNA synthesis, we demonstrate that it is not required for other PDGF-induced cellular responses such as PLC and PKC activation or PDGF B chain gene induction. These data suggest that mevalonate starvation likely influences a late step in the PDGF mitogenic pathway.

In summary, we have demonstrated a potential beneficial effect of an HMGCoA reductase inhibitor on mesangial cells. These *in vitro* studies suggest that some of the effects of HMGCoA reductase inhibitors *in vivo* to ameliorate experimental glomerular injury could be mediated, at least in part, by a direct effect on glomerular cells. The ameliorative effect of lovastatin on mesangial cell proliferation without pronounced reduction in serum cholesterol levels in a model of glomerulosclerosis accelerated by dietary lipid supplementation supports this hypothesis [33]. A recent study in human subjects with hypertension and chronic renal failure demonstrated a superior beneficial effect of combination therapy with simvastatin and angiotensin converting enzyme inhibition over therapy with angiotensin converting enzyme inhibition alone in preserving renal function [34]. Further studies will be necessary to determine if HMGCoA inhibitors exert additional effects on target cells besides inhibition of mitogenesis.

Acknowledgments

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